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Metabolic inhibitors, elicitors, and precursors as tools for probing yield limitation in taxane production by Taxus chinensis cell cultures
AU Srinivasan, V.; Ciddi, V.; ***Bringi, V.*** ; Shuler, M. L.
CS School of Chemical Engineering, Cornell University, Ithaca, NY, 14853, USA
SO Biotechnol. Prog. (1996), 12(4), 457-465

Large scale production of secondary metabolites using plant cell cultures: Opportunities, realities and challenges.
AU Venkat, K.; ***Bringi, V.*** ; Kadkade, P.; Prince, C.
CS Phyton Inc., Itacha, NY 14850 USA
SO Abstracts of Papers American Chemical Society, (1997) Vol. 213, No. 1-3, pp. AGFD 54.
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I Large scale production of secondary metabolites using plant cell cultures: Opportunities, realities and challenges
AU Venkat, K.; ***Bringi, V.*** ; Kadkade, P.; Prince, C.
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SO Book of Abstracts, 213th ACS National Meeting, San Francisco, April 13-17 (1997), AGFD-054 Publisher: American Chemical Society, Washington, D. C.

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Production of ***taxol*** by cell culture of Taxus. For development of techniques for industrial production
AU Hara, Yasuhiro; Yukimune, Yukihito
CS Mitsui Petrochem. Ind., Ltd., Yamaguchi, 740, Japan
SO Farumashia (1996), 32(7), 806-809
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LA Japanese

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TI Effect of picloram and methyl ***jasmonate*** on growth and ***taxane*** accumulation in callus culture of Taxus X media var. Hatfieldii.
AU Furmanowa, M.; Glowniak, K.; Syklowska-Baranek, K.
SO Plant cell, tissue and organ culture, 1997. Vol. 49, No. 1. p. 75-79
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TI Large-scale plant cell culture
AU Roberts, Susan C.; Shuler, Michael L.
CS Sch. Chemical Eng., Cornell Univ., Ithaca, NY, 14853-5201, USA
SO Curr. Opin. Biotechnol. (1997), 8(2), 154-159

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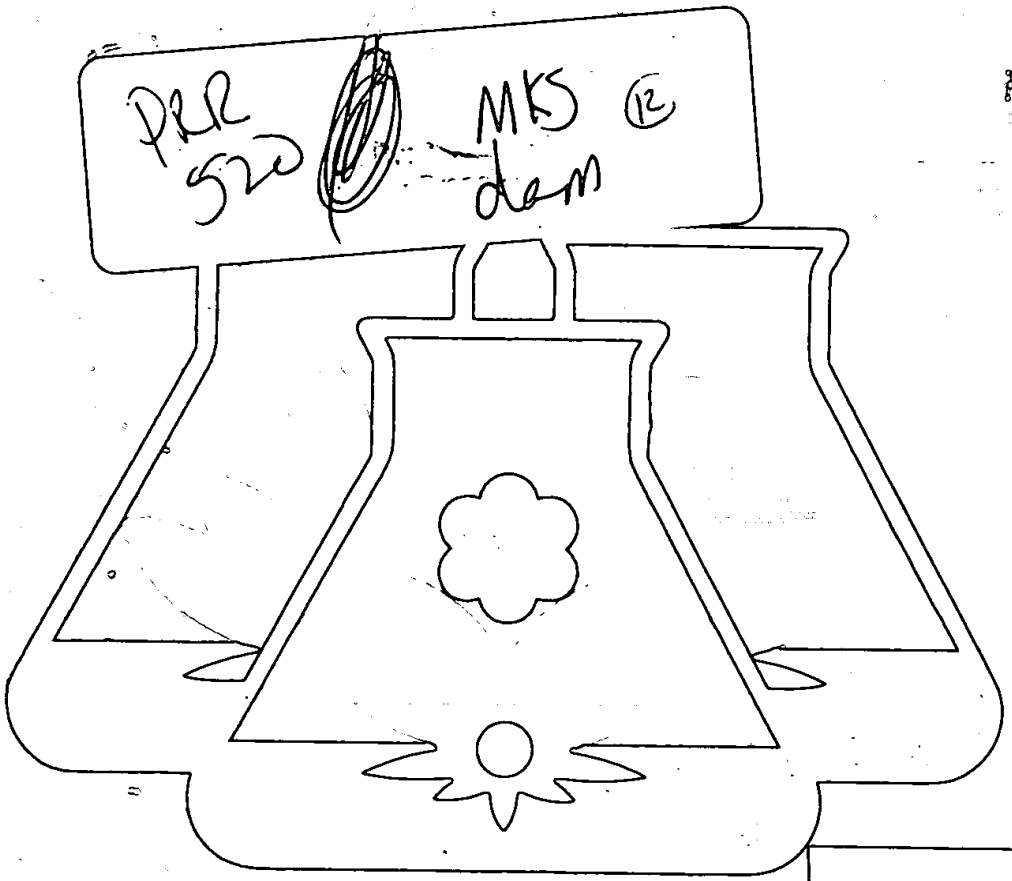

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Research note

Effect of picloram and methyl jasmonate on growth and taxane accumulation in callus culture of *Taxus* × *media var. Hatfieldii*

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Abstract

We have analysed the effect of some culture conditions and media components on callus growth rate and production of taxanes in callus of *Taxus* × *media var. Hatfieldii*. For callus induction and maintenance a Gamborg B5 medium and a White - Rangaswamy medium (WR) with different modifications were used. On an improved WR medium (containing 10 μM picloram) the callus growth factor increased up to 5.8 fold (fresh weight). Picloram only enhanced the growth of callus, but not taxane production. On WR medium with (100 μM) methyl jasmonate the paclitaxel content increased from 2.37 $\mu g\ g^{-1}$ to 90 $\mu g\ g^{-1}$ and cephalomannine from 5.14 $\mu g\ g^{-1}$ to 29.14 $\mu g\ g^{-1}$ (dry weight), whereas growth of the cultures ceased. The presence of paclitaxel and cephalomannine was established by high performance liquid chromatography.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; B5 – Gamborg's B5 medium; NAA – α-naphthaleneacetic acid; WR – modified White medium (Rangaswamy, 1961)

Taxus species (Taxaceae, Gymnosperms) contain many taxane diterpenes, among them paclitaxel (earlier known as taxol) which plays a most interesting role as an antitumor drug. The antitumorous properties of paclitaxel are based on the ability to bind and to stabilize microtubules and block cell replication in the late G2-M phase of the cell cycle (Liebmann et al., 1994). Taxol® (containing paclitaxel) was approved in 1992 by the US Food and Drug Administration for the treatment of ovarian and breast cancer. This drug was also registered in Poland in 1996.

Among the many T*axus* species in which paclitaxel was determined is *Taxus* × *media* Rehd. and its varieties: *Taxus* × *media var Hicksii, Brownii, Densiformis, Fieldii, Nigra, Hatfieldii* (Wickremesinhe and Arteca, 1993; Matysik et al., 1995). Part of the paclitaxel is localised on the surface of the needles of there Taxus species (Zobel et al., 1996). In callus of two varieties, *T. x m. var. Hicksii* and *T. x m. var. Hatfieldii*, paclitaxel and other taxanes were found (Furmanowa et al., 1995a).

Here we report preliminary observations the effects of medium composition, growth conditions and time of culture on callus induction, growth of tissue and production of taxanes in callus of *Taxus x media var. Hatfieldii*.

The twigs of a male tree *Taxus* × *media var. Hatfieldii* were collected in the Botanical Garden of the Polish Academy of Sciences in Warsaw. The voucher specimen which were used for phytochemical analysis and callus induction is kept at the Herbarium of the Department of Biology and Pharmaceutical Botany, Warsaw Medical Academy, Warsaw. The twigs were collected in different seasons spring (May), autumn (September) and winter (January). Twigs were washed in distilled water, immersed in 70% ethanol for 1 min., surface sterilized by immersion in 2.5% sodium hypochlorite (NaC10) for 30 min and rinsed three

times with sterile distilled water. Explants (stem segments and needles, 1 to 2 cm long) were transferred to too solid media (Furmanowa et al., 1995b) a modified White medium according to Rangaswamy (WR) (1961) and Gamborg's B5 (1968) supplemented with different growth regulators (2,4-D, NAA, picloram). Methyl jasmonate (100 μM) was added to the corresponding media after autoclaving. The pH of the medium was adjusted to 5.6 with 1N potassium hydroxide. Cultivation was carried out in 100 ml Erlenmeyer flasks under a light regime of: 18h light (40 μmol m^{-2} s^{-1}) and 6h dark. In some experiments callus was cultivated only in the dark or only in the light. One passage was 8 week long. Biomass increase was calculated always after eight weeks of culture. The cytomorphological observation of callus growth on different media modifications was done after each passage. For chemical analysis callus samples were lyophilised.

Statistical analyses (ten samples for each treatment) were performed by one-way ANOVA with multiple dependent measures MANOVA (in case of the effect of time of plant collection and culture conditions on callus growth). For two treatment comparisons standard deviations were calculated using t-tests. Significance level for all statistical tests was 5%.

Acidified methanol extracts from lyophilised tissue and ground organs were passed through C-18 Baker Bond (J.T.Baker Inc.) - SPE columns (3 ml of packing volume) and eluated with 70% methanol. Eluates containing paclitaxel (confirmed by TLC) were analysed by HPLC. The HPLC analysis was carried out using a Hewlett Packard (H-P) Model 1050 Liquid chromatograph with a 20 μl sample injector (Rheodyne, Cotati, CA, USA) and a spectrophotometric UV-VIS detector. The chromatograms were recorded at 200 nm with 3396A reporting integrator (H-P). A stainless steel column 200 × 4.6 mm I.D., packed with 5 μm ODS Hypersil (H-P) was used. 10 μl samples were injected. As a mobile phase acetonitrile/water (1/1 v/v) was used at a flow rate of 1 ml min^{-1} and a temperature of 20°C. As standard substances paclitaxel and cephalomannine (obtained from Trent University Peterborough, Ontario, Canada) were used.

The needles of *Taxus* × *media var. Hatfieldii* used for callus induction contained paclitaxel in the range 357.14 μg g^{-1} to 773.65 μg g^{-1} and cephalomannine in the range 184.62 μg g^{-1} to 357.69μg g^{-1}. The most interesting observation for callus cultures of *Taxus* × *media var. Hatfieldii* was the influence of picloram on callus growth and the stimulatory effect of methyl jasmonate on paclitaxel and cephalomannine production in the callus cultured on WR medium (Table 1).

Picloram (4-amino-3,5,6-trichloropicolinic acid) is not commonly used for plant tissue culture. In yew tissue culture picloram was used by Bringi et al. (1995), who presented a novel process for recovering taxanes in high yields from *Taxus* sp., but no details on the effect of picloram on growth rates were given. The effect of picloram also studied by Ketchum and Gibson (1996). In our observation 10 μM picloram caused better growth of callus on WR and B5 media, cultivated in the light as well as in the dark, but the effect of picloram was most marked on WR medium in the light (11.4-fold). In the dark callus also grew well (9.3-fold) .Without picloram the callus growth was only 0.9-fold. In the light the average growth of callus, on media containing picloram, was two to four-fold higher in comparison with control medium (Figure 1). This auxin had no effect on taxane production (Table 1). The high growth rate was not stable.

The highest growth rate of callus derived from needles collected during the winter (January and February) was usually in the second passage, but for cultures derived from needles collected in May in later (fourth and fifth) passages.

Jasmonates showed various morphological and physiological activities when exogenously applied to plants. The jasmonates are possibly signal compounds in the elicitation process leading to *de novo* transcription and translation and ultimately, to the biosynthesis of secondary metabolites in plant cell cultures. They induced transcriptional activation of genes involved in the formation of secondary metabolites (Yukimune et al., 1996). Jasmonic acid was used for the production of taxane diterpenes in tissue culture of many *Taxus* species and the cytostatic agents can be produced in high yield on an industrial scale (Yukimune et al., 1995). At the same time Furmanowa et al. (1995b) found also that methyl jasmonate stimulated the paclitaxel and cephalomannine production in callus and suspension culture of *Taxus baccata*. Mirjalili and Linden (1996) examined the influence of different concentrations and combination of methyl jasmonate and ethylene on paclitaxel production. The highest content was 3.4 mg l^{-1} and 2.7 mg l^{-1} after elicitation with 10 μM methyl jasmonate and 5 ppm of ethylene, and 10 μM methyl jasmonate and 0 ppm ethylene, respectively. Our present results show that WR medium supplemented with 100 μM methyl jasmonate significantly stimulated the paclitaxel content in callus of *Taxus* × *media var. Hatfieldii*. Yukimune et al. (1996) obtained the

Table 1. Growth factor and taxane production in callus of *Taxus* × *media var. Hatfieldii*.

Date of collection	Medium	Growth regulators	Source of explant	No. of passages	Growth conditions	Callus growth rate (CGF - x-fold)	Contents (dry wt.) paclitaxel $\mu g\ g^{-1}$	cephalomannine $\mu g\ g^{-1}$
Winter	WR	Picloram 5 μM NAA 10.5 μM + methyl jasm 100 μM	twigs	I	dark	0.1	60.00	77.14
	WR	Picloram 5 μM NAA 10.5 μM	needles	I	dark	4.3	5.15	13.04
	B5	Picloram 5 μM NAA 10.5 μM	needles	I	light	5.8	traces	traces
	WR	2,4-D 22.6μM + 100 μM methyl jasm.	needles	VIII	dark	0.03	89.98	2.76
	WR	2,4-D 22.6 μM	needles	VIII	dark	1.8	2.37	3.43
Spring	WR	2,4-D 22.6 μM	needles	VI	light	5.0	traces	5.14
	WR	2,4-D 22.6 μM + 100 μM methyl jasm.	needles	VI	light	0.05	85.26	29.14
	WR	2,4-D 22.6 μM	twigs	VI	light	2.2	traces	6.86
Autumn	B5	NAA 10.5 μM + 100 μM methyl jasm.	twigs	IV	light	0.04	traces	traces
	B5	NAA 10.5 μM + 100 μM methyl jasm.	twigs	IV	dark	0.08	traces	traces

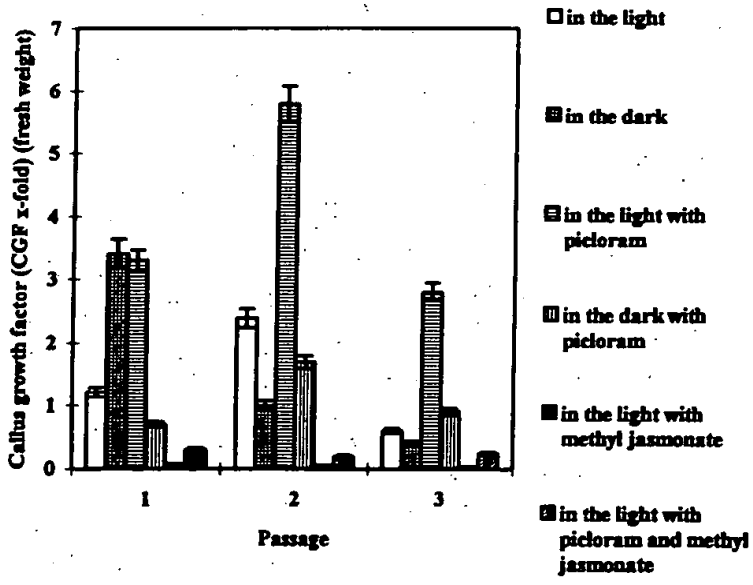

Figure 1. Growth of needle-derived callus (needles collection - winter) of *Taxus* × *media var. Hatfieldii* on B5 medium with NAA 2 mg l^{-1} under different conditions at 3 passages.

highest paclitaxel accumulation (104.2 mg l^{-1}) when 100 μM of methyl jasmonate was added to the medium.

In our experiments the content of paclitaxel increased from 2.37 $\mu g\ g^{-1}$ to 89.98 $\mu g\ g^{-1}$ in callus growing on WR medium supplemented with 100 μM

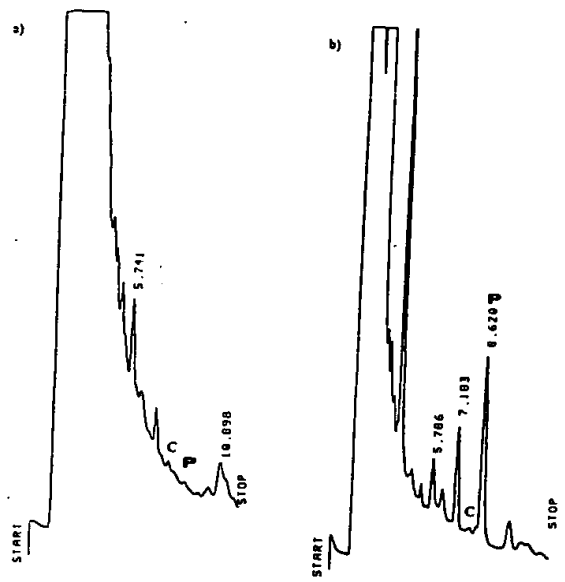

Figure 2. Chromatograms of methanolic extracts from needle-derived callus of *Taxus* × *media var. Hatfieldii* cultivated on WR medium with growth regulators: (*a*) 2,4-D (in dark); (*b*) 2,4-D + methyl jasmonate (in dark). C-cephalomannine; P-paclitaxel.

methyl jasmonate (Figure 2). Using B5 medium only traces of paclitaxel and cephalomannine were found. Methyl jasmonate had no effect on taxane production on B5 medium in our experiments (Table 1). It was very interesting to find that methyl jasmonate almost completely ceased (on WR media) growth of callus. Similar growth inhibition was observed on B5 medium (Table 1). In our experiments the WR medium is much better for taxane production than B5. It might be caused by reduced concentration of $KNO_3$and elimination of $(NH_4)_2SO_4$ from the WR medium. Our observations are in accordance to Yukimune et al. (1996) who studied three *Taxus* species and stated that treatment with methyl jasmonate decreased the cell yield but increased taxane contents. We also found that in this condition paclitaxel productivity is higher than cephalomannine one (Figure 2).

On the base of our observations for taxane production the use of a two stage culture method is very helpful: for growth of callus - WR medium supplemented with picloram, and for taxane production WR medium with methyl jasmonate. From our point of view it is easier to elaborate the two stage culture conditions than to find the medium on which the growth of cells and paclitaxel formation are not inversely related (Ketchum and Gibson, 1996).

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